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Genetic structure of *Plasmodium falciparum* populations between lowland and highland sites and antimalarial drug resistance in Western Kenya

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ABSTRACT

Human travel to malaria endemic lowlands from epidemic highlands has been shown to increase the risk of malaria infections in the highlands. In order to gain insight on the impact of human travel, we examined prevalence, genetic variability and population genetic structure of *Plasmodium falciparum* in asymptomatic children from one highland site and three surrounding malaria endemic lowland sites in Western Kenya, using multilocus microsatellite genotyping. We further analyzed the frequencies of mutations at the genes conferring resistance to chloroquine and sulfadoxine–pyrimethamine. We found a significant decrease in malaria prevalence in the highland site from 2006 to 2007, 1 year after the introduction of the artemisinin-based combination therapy as first-line treatment for uncomplicated malaria and the scale-up of insecticide-treated bed nets. Population genetic diversity, measured by the number of observed and effective microsatellite alleles and Nei's unbiased genetic diversity, was high and comparable for both highland and lowland populations. Analysis of molecular variance did not detect a significant genetic structure across highland and lowland regions. Similarly, mutations at key antimalarial-resistance codons of the *pfcrt*, *pfmdr1*, *pfdhfr* and *pfdhps* genes were found at comparable high frequencies in all four sites. High level of gene flow and lack of significant genetic structure in malaria parasites between highland and lowland areas suggest the importance of human travel in shaping parasite population structure.

1. Introduction

Malaria remains the leading cause of morbidity and mortality in Africa, with an estimated death toll exceeding one million people each year (WHO, 2000). Stable endemic malaria thrives throughout the African continent, but epidemic malaria occurs at the fringe of the endemic areas, particularly in areas of higher altitudes, in the arid regions of North Africa and in communities living at the most southern latitudes (Hay et al., 2002). The densely populated and agriculturally significant highlands of Western Kenya are among these epidemic prone-areas. In the past century, the highlands of Western Kenya suffered from two periods of epidemic malaria. The first occurred in the early 1930s and was contained by the 1960s through the use of indoor residual spray with DDT and mass-drug administration (Shanks et al., 2005b). However, epidemics have resurged since the late 1980s (Shanks et al., 2005a). A number of hypotheses have been proposed to explain the mechanisms for the re-emergence of malaria in African highlands: among these are antimalarial drug resistance (Zhong et al., 2008) and parasite introduction from surrounding endemic lowlands through human travel (Shanks et al., 2005a).

Currently, the central tools of malaria prevention and treatment are insecticide-treated bed nets (ITNs) and artemisinin-based combination therapy (ACT). ACT, in the form of artemether–lumefantrine combination, has replaced sulfadoxine–pyrimethamine (SP) and since 2006 has become the first-line antimalarial drug in rural Kenya communities (Amin et al., 2007). Nonetheless, SP is still used for intermittent preventive treatment in pregnancy while quinine is the drug of choice for treating children <5 kg and pregnant women (Zurovac et al., 2008). Despite the fact that human factors, such as the immune response, influence treatment outcome (White, 2002), indications of the level of resistance to antimalarial drugs can be estimated from the frequency of resistance allele types in known drug target genes. Specifically, codon 76 of the chloroquine resistance transporter gene (*pfcrt*) and codons 86, 1042, and 1246 of the *Plasmodium falciparum* multidrug

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resistance gene 1 (*pfmdr1*) are markers for resistance to chloroquine (CQ) (Babiker et al., 2001; Dorsey et al., 2001). The *pfcrt* (K76T) mutation is essential to CQ resistance (Babiker et al., 2001; Dorsey et al., 2001), but mutations in the *pfmdr1* gene modulate the degree of CQ resistance (Sanchez and Lanzer, 2000) and are also thought to play a role in lumefantrine resistance (Sisowath et al., 2005). Mutations at codons 437 and 540 of the dihydropteroate synthetase (*dhps*) gene and at codons 51, 59, and 108 of the dihydrofolate reductase (*dhfr*) gene are markers for resistance to SP (Ouellette, 2001; Peterson et al., 1990). Clinical studies show that SP treatment failure is associated with the quintuple mutant haplotype *dhfr*-108N/51I/59R *dhps*-437G/540E (Kublin et al., 2002) and begins to appear with the triple mutant haplotype *dhfr*-108N/51I/59R (Talisuna et al., 2003). Mutations for resistance to the artemisinin derivates have not been identified yet (Nosten and White, 2007).

In this paper, we examined the level of genetic polymorphism of asymptomatic *P. falciparum* infections from three lowland localities and one highland site in Western Kenya, and the frequencies of gene mutations for SP and CQ resistance. Our goal is to determine the genetic structure and gene flow of malaria parasite populations, and to provide baseline mutation frequencies at the onset of large-scale ACT application for malaria control. The baseline mutation frequency information is useful in tracking the evolution of genes associated with SP and CQ resistance when SP and CQ selection pressure on malaria parasites is relaxed, and in the deployment of appropriate antimalarial drugs (Laufer et al., 2007).

2. Materials and methods

2.1. Study sites and sample collection

As a part of malaria surveillance activities, blood samples were taken from a total of 599 primary school children (age 6–14) in three lowland sites: Chemelil (35°08″E, 0°05″S, altitude 1248 m), Miwani (34°58″E, 0°03″S, altitude 1214 m) and Kisian (34°40″E, 0°04″S, altitude 1164 m); and in the village of Iguhu, in the highland district of Kakamega (34°35°E, 0°010″S, altitude 1480–1580 m) (Fig. 1). Chemelil and Miwani were sampled in November 2006; Kisian was sampled in October 2006. Iguhu was sampled in October 2006 and June 2007; hereafter the 2006 sample is referred as Iguhu06, the 2007 as Iguhu07. We chose these three representative lowland sites because there is extensive human travel to and from the highland site. If the lowland sites are the source of infection to the highland site, these lowland sites could best represent the main source of infection to the highland site. However, other lowland sites that were not sampled in the present study could also be a source of infection to the highland site.

Malaria transmission in the lowland sites is perennial, with the main vectors being *A. gambiae* s.s., *A. arabiensis* and *A. funestus* (Githeko et al., 1996; Mutuku et al., 2006). Chemilil and Miwani are within Nyando district, where the temperature ranges from 16.2 to 29.3 °C. Average annual rainfall in these two sites is about 1600 mm. Kisian is a village on the shores of Lake Victoria in Kisumu district with temperature ranging from 15.0 to 28.4 °C and an average annual rainfall of 1400 mm during the period 1970–2000. In this site, the entomological inoculation rate (EIR) was estimated at 31.1 infectious bites per person per year in 2003–2004 (Ndenga et al., 2006) and the average malaria prevalence among primary school children during the sampling period was 55%. The village of Iguhu experiences two rainy seasons and averages about 1800 mm rainfall per year. The long rainy season usually occurs between mid-March and May while the short rainy season occurs between September and October. The mean annual temperature is 20.8 °C. The predominant malaria vector species in the area is *A. gambiae* s.s. EIR has been estimated at 16.6 and malaria prevalence at 34% in 2003–2004 (Ndenga et al., 2006).

From each child, approximately 200 μl of finger-pricked blood was spotted onto a filter paper, air-dried and stored at −20 °C until parasite DNA extraction. *P. falciparum* asymptomatic infections were diagnosed by microscopy as previously reported (Munyekenye et al., 2005). The human subject protocol involved in this study was approved by the University of California at Irvine, USA and The Kenya Medical Research Institute, Kenya.

2.2. Parasite DNA extraction and species identification

DNA was extracted from the blood-filters using the Saponin/Chelex method (Wooden et al., 1993). Parasite DNA was extracted from one quarter of a blood spot of about 1 cm in diameter and dissolved in ~200 μl of distilled water. *P. falciparum* infections were identified by a species-specific nested PCR method as previously described (Singh et al., 1999). Only samples positive for *P. falciparum* DNA were used for genotyping analysis.

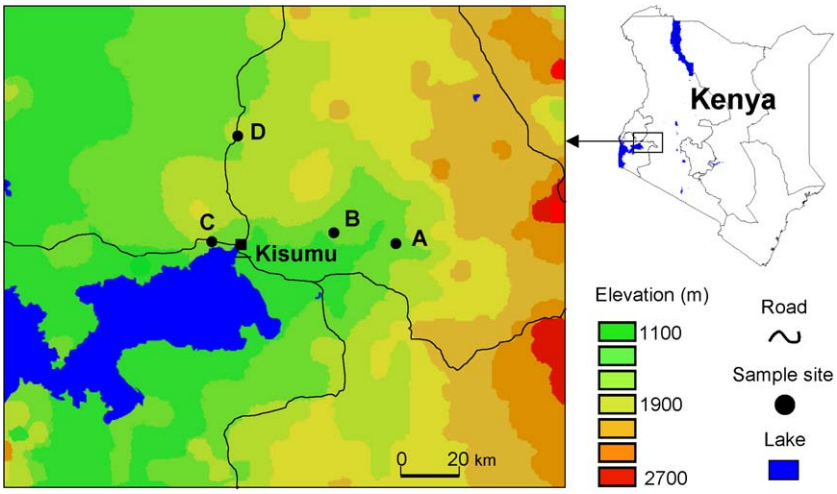

Fig. 1. A map showing the distribution of sampling sites in Nyanza and Western provinces of Kenya. The three lowland sites are: Chemelil (A), Miwani (B), and Kisian (C). The highland site is Iguhu (D) in the Kakamega district. A total of 599 asymptomatic children (age 6–14 years), including 299 from the lowlands and 300 from the highlands, were enrolled in this study.